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PATENT APPLICATION

NOVEL HUMAN CALCIUM CHANNELS AND RELATED PROBES, CELL LINES AND METHODS

This application is a continuation-in-part of copending US Patent Application No. Serial No. 09/030,482, filed February 25, 1998, which is a 111(a) application claiming priority from US Provisional Application No. 60/039,204, filed February 28, 1997, both of which are incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to novel mammalian (including human) calcium channel compositions, and to the expression of these compositions in cell lines for use in evaluating calcium channel function and the behavior of compositions which modulate calcium channel function.

BACKGROUND OF THE INVENTION

The rapid entry of calcium into cells is mediated by a class of proteins called voltage-gated calcium channels. Calcium channels are a heterogeneous class of molecules that respond to depolarization by opening a calcium-selective pore through the plasma membrane. The entry of calcium into cells mediates a wide variety of cellular and physiological responses including excitation-contraction coupling, hormone secretion and gene expression. In neurons, calcium entry directly affects membrane potential and contributes to electrical properties such as excitability, repetitive firing patterns and pacemaker activity. Miller, R.J. (1987) "Multiple calcium channels and neuronal function." *Science* 235:46-52. Calcium entry further affects neuronal functions by directly regulating calcium-dependent ion channels and modulating the activity of calcium-dependent enzymes such as protein kinase C and calmodulin-dependent protein kinase II. An increase in calcium concentration at the presynaptic nerve terminal triggers the release of neurotransmitter. Calcium entry also plays a

role in neurite outgrowth and growth cone migration in developing neurons and has been implicated in long-term changes in neuronal activity.

In addition to the variety of normal physiological functions mediated by calcium channels, they are also implicated in a number of human disorders. Recently, mutations identified in human and mouse calcium channel genes have been found to account for several disorders including, familial hemiplegic migraine, episodic ataxia type 2, cerebellar ataxia, absence epilepsy and seizures. Fletcher, et al. (1996) "Absence epilepsy in tottering mutant mice is associated with calcium channel defects." *Cell* 87:607-617; Burgess, et al. (1997) "Mutation of the Ca2+ channel β subunit gene Cchb4 is associated with ataxia and seizures in the lethargic (lh) mouse." *Cell* 88:385-392; Ophoff, et al. (1996) "Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca2+ channel gene CACNL1A4." *Cell* 87:543-552; Zhuchenko, O. et al. (1997) "Autosomal dominant cerebellar ataxia (SCA6) associated with the small polyglutamine expansions in the α1A-voltage-dependent calcium channel." *Nature Genetics* 15:62-69.

The clinical treatment of some disorders has been aided by the development of therapeutic calcium channel antagonists. Janis, et al. (1991) in *Calcium Channels: Their Properties, Functions, Regulation and Clinical Relevance*. CRC Press, London.

Native calcium channels have been classified by their electrophysiological and pharmacological properties as T, L, N, P and Q types (for reviews see McCleskey, et al. (1991) "Functional properties of voltage-dependent calcium channels." *Curr. Topics Membr.* 39: 295-326, and Dunlap, et al. (1995) "Exocytotic Ca^{2+} channels in mammalian central neurons." *Trends Neurosci.* 18:89-98.). T-type (or low voltage-activated) channels describe a broad class of molecules that activate at negative potentials and are highly sensitive to changes in resting potential. The L, N, P and Q-type channels activate at more positive potentials and display diverse kinetics and voltage-dependent properties. There is some overlap in biophysical properties of the high voltage-activated channels, consequently pharmacological profiles are useful to further distinguish them. L-type channels are sensitive

to dihydropyridine (DHP) agonists and antagonists, N-type channels are blocked by the *Conus geographus* peptide toxin, ω-conotoxin GVIA, and P-type channels are blocked by the peptide ω-agatoxin IVA from the venom of the funnel web spider, *Agelenopsis aperta*. A fourth type of high voltage-activated Ca channel (Q-type) has been described, although whether the Q- and P-type channels are distinct molecular entities is controversial (Sather et al. (1993) "Distinctive biophysical and pharmacological properties of class A (B1) calcium channel α1 subunits." *Neuron* 11: 291-303; Stea, et al. (1994) "Localization and functional properties of a rat brain α1A calcium channel reflect similarities to neuronal Q- and P-type channels." *Proc Natl Acad Sci (USA)* 91: 10576-10580.). Several types of calcium conductances do not fall neatly into any of the above categories and there is variability of properties even within a category suggesting that additional calcium channels subtypes remain to be classified.

Biochemical analyses show that neuronal high-threshold calcium channels are heterooligomeric complexes consisting of three distinct subunits (α_1, $\alpha_2\delta$ and β)(reviewed by De Waard, et al. (1997) in *Ion Channels*, Volume 4, edited by Narahashi, T. Plenum Press, New York). The α_1 subunit is the major pore-forming subunit and contains the voltage sensor and binding sites for calcium channel antagonists. The mainly extracellular α_2 is disulphide-linked to the transmembrane δ subunit and both are derived from the same gene and are proteolytically cleaved *in vivo*. The β subunit is a non-glycosylated, hydrophilic protein with a high affinity of binding to a cytoplasmic region of the α_1 subunit. A fourth subunit, γ, is unique to L-type Ca channels expressed in skeletal muscle T-tubules. The isolation and characterization of γ-subunit-encoding cDNAs is described in US Patent No. 5,386,025 which is incorporated herein by reference.

Molecular cloning has revealed the cDNA and corresponding amino acid sequences of six different types of α_1 subunits (α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E} and α_{1S}) and four types of β subunits (β_1, β_2, β_3 and β_4)(reviewed in Stea, A., Soong, T.W. and Snutch, T.P. (1994) "Voltage-gated calcium channels." in *Handbook of Receptors and Channels*. Edited by R.A. North, CRC

Press.). PCT Patent Publication WO 95/04144, which is incorporated herein by reference, discloses the sequence and expression of α_{1E} calcium channel subunits.

The different classes of $\alpha 1$ and β subunits have been identified in different animals including, rat, rabbit and human and share a significant degree of amino acid conservation across species (for examples see: Castellano, et al. (1993) "Cloning and expression of a third calcium channel β subunit." *J. Biol. Chem.* 268: 3450-3455; Castellano, et al. (1993) "Cloning and expression of a neuronal calcium channel β subunit." *J. Biol. Chem.* 268: 12359-12366; Dubel, et al. (1992). "Molecular cloning of the α_1 subunit of an ω-conotoxin-sensitive calcium channel." *Proc. Natl. Acad. Sci. (USA)* 89: 5058-5062; Fujita, et al.. (1993) "Primary structure and functional expression of the ω-conotoxin-sensitive N-type calcium channel from rabbit brain." *Neuron* 10: 585-598; Mikami, et al.. (1989). "Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel." *Nature* 340: 230-233; Mori, et al. (1991) "Primary structure and functional expression from complementary DNA of a brain calcium channel." *Nature* 350: 398-402; Perez-Reyes, et al. (1992). "Cloning and expression of a cardiac/brain ß subunit of the L-type calcium channel." *J. Biol. Chem.* 267: 1792-1797; Pragnell, et al. (1991). "Cloning and tissue-specific expression of the brain calcium channel β-subunit." *FEBS Lett.* 291: 253-258; Snutch, et al. (1991) "Distinct calcium channels are generated by alternative splicing and are differentially expressed in the mammalian CNS." *Neuron* 7: 45-57; Soong, et al. (1993) "Structure and functional expression of a member of the low voltage-activated calcium channel family." *Science* 260: 1133-1136; Tomlinson, et al. (1993) "Functional properties of a neuronal class C L-type channel." *Neuropharmacology* 32: 1117-1126; Williams, et al. (1992) "Structure and functional expression of $\alpha 1$, $\alpha 2$, and ß subunits of a novel human neuronal calcium channel subtype." *Neuron* 8: 71-84; Williams, et al. (1992) "Structure and functional expression of an ω-conotoxin-sensitive human N-type calcium channel." *Science* 257: 389-395.

In some expression systems the α_1 subunits alone can form functional calcium channels although their electrophysiological and pharmacological properties can be differentially modulated by coexpression with any of the four β subunits. Until recently, the reported modulatory affects of β subunit coexpression were to mainly alter kinetic and voltage-dependent properties. More recently it has been shown that β subunits also play crucial roles in modulating channel activity by protein kinase A, protein kinase C and direct G-protein interaction. (Bourinet, et al. (1994) "Voltage-dependent facilitation of a neuronal α1C L-type calcium channel." *EMBO J.* 13: 5032-5039; Stea, et al. (1995) "Determinants of PKC-dependent modulation of a family of neuronal calcium channels." *Neuron* 15:929-940; Bourinet, et al. (1996) "Determinants of the G-protein-dependent opioid modulation of neuronal calcium channels." *Proc. Natl. Acad. Sci. (USA)* 93: 1486-1491.)

The electrophysiological and pharmacological properties of the calcium channels cloned to date can be summarized as shown in Table 1. While the cloned α_1 subunits identified to date correspond to several of the calcium channels found in cells, they do not account for all types of calcium conductances described in native cells. For example, they do not account for the various properties described for the heterogenous family described as T-type calcium channels. Furthermore, they do not account for novel calcium channels described in cerebellar granule cells or other types of cells. (Forti, et al (1993) "Functional diversity of L-type calcium channels in rat cerebellar neurons." *Neuron* 10: 437-450; Tottene,et al. (1996). "Functional diversity of P-type and R-type calcium channels in rat cerebellar neurons." *J. Neurosci.* 16: 6353-6363).

Because of the importance of calcium channels in cellular metabolism and human disease, it would be desirable to identify the remaining classes of α_1 subunits, and to develop expression systems for these subunits which would permit the study and characterization of these calcium channels, including the study of pharmacological modulators of calcium channel function. Thus, it is an object of the present invention to provide heretofor undisclosed calcium channels having novel α_1 subunits, including cell lines expressing these

TABLE 1

	ω-conotoxin GVIA	1,4-dihydropyridines	cadmium	ω-agatoxin IVA	ω-conotoxin MVIIC	native Ca^{2+} channel type
α_{1A}	-	-	✓	✓	✓	P/Q-type
α_{1B}	✓	-	✓	-	✓	N-type
α_{1C}	-	✓	✓	-	-	L-type
α_{1D}	-	✓	✓	-	-	L-type
α_{1E}	-	-	✓	-	-	novel
α_{1S}	-	✓	✓	-	-	L-type

new calcium channels. It is a further object of the present invention to provide a method for testing these novel calcium channels using such cell lines.

SUMMARY OF THE INVENTION

The present invention provides sequences for a novel mammalian calcium channel subunits of T-type calcium channels, which we have labeled as α_{1G}, α_{1H} and α_{1I} subunits. Knowledge of the sequences of these calcium channel subunits may be used in the development of probes for mapping the distribution and expression of the subunits in target tissues. In addition, these subunits, either alone or assembled with other proteins, can produce functional calcium channels, which can be evaluated in model cell lines to determine the properties of the channels containing the subunits of the invention. These cell lines can be used to evaluates the affects of pharmaceuticals and/or toxic substances on calcium channels incorporating α_{1G}, α_{1H} and α_{1I} subunits

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and B show a comparison of the waveforms and current voltage relationship for α_{1G};

Figs. 2A and Bshow a comparison of the waveforms and current voltage relationship for α_{1I} calcium channels.

Fig. 3 shows a comparison of the steady state inactivation profiles of the α_{1G} and α_{1I} calcium channels.

Figs. 4A-C show a comparison of the inactivation kinetics of the α_{1G} and α_{1I} calcium channels.

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes the following aspects for which protection is sought:

(a) novel mammalian (including human) calcium channel subunits and DNA sequences encoding such subunits. Specifically, the invention encompasses an at least partially purified DNA molecule comprising a sequence of nucleotides that encodes an α subunit of a T-type calcium channel, and such α subunits *per se*. It will be appreciated that polymorphic variations may be made or may exist in the DNA of some individuals leading to minor deviations in the DNA or amino acids sequences from those shown which do not lead to any substantial alteration in the function of the calcium channel. Such variations, including variations which lead to substitutions of amino acids having similar properties are considered to be within the scope of the present invention. Thus, in one embodiment, the present application claims DNA molecules which encode α_1 subunits of mammalian T-type calcium channels, and which hybridize under conditions of medium (or higher) hybridization stringency with one or another of the specific sequences disclosed in this application. This level of hybridization stringency is generally sufficient given the length of the seqeunces involved to permit recovery of the subunits within the scope of the invention from mammalian DNA libraries.

(b) polynucleotide sequences useful as probes in screening human cDNA libraries for genes encoding these novel calcium channel subunits. These probes can also be used in histological assay to determine the tissue distribution of the novel calcium channel subunits.

(c) at least partially purified α_1 subunits and related peptides for mammalian T-type calcium channels. These proteins and peptides can be used to generate polyclonal or monoclonal antibodies to determine the cellular and subcellular distribution of T-type calcium channel subunits.

(d) eukaryotic cell lines expressing the novel calcium channel subunits. These cell lines can be used to evaluate compounds as pharmacological modifiers of the function of the novel calcium channel subunits.

(e) a method for evaluating compounds as pharmacological modifiers of the function of the novel calcium channel subunits using the cell lines expressing those subunits alone or in combination with other calcium channel subunits.

Further, since defects in the novel calcium channel subunits may be associated with a human genetic disease including, but not limited to; epilepsy, migraine, ataxia, schizophrenia, hypertension, arrhythmia, angina, depression, small lung carcinoma, Lambert-Eaton syndrome and Parkinson's disease; characterization of such associations and ultimately diagnosis of associated diseases can be carried out with probes which bind to the wild-type or defective forms of the novel calcium channels.

As used in the specification and claims of this application, the term "T-type calcium channel" refers to a voltage-gated calcium channel having a low activation voltage, generally less than -50 mV, and most commonly less than -60 mV. T-type calcium channels also exhibit comparatively negative steady-state inactivation properties and slow deactivation kinetics. The terms "α_1 subunit" or "α_1 calcium channel" refer to a protein subunit of a calcium channel which is responsible for pore formation and contains the voltage sensor and binding sites for calcium channel agonists and antagonists. Such subunits may be independently functional as calcium channels or may require the presence of other subunit types for complete functionality.

As used in the specification and claims of this application, the phrase "at least partially purified" refers to DNA or protein preparations in the which the specified molecule has been separated from adjacent cellular components and molecules with which it occurs in the natural state, either by virtue of performing a physical separation process or by virtue of making the DNA or protein molecule in a non-natural environment in the first place. The term encompasses cDNA molecules and expression vectors.

In accordance with the present invention, we have identified mammalian DNA sequences which code for novel T-type calcium channel α_1 subunits. These subunits are

believed to represent new types of α_1 subunits of mammalian voltage-dependent calcium channels which have been designated as types α_{1G}, α_{1H} and α_{1I}.

The novel α_1 subunits of the invention were identified by screening the *C. elegans* genomic DNA sequence data base for sequences homologous to previously identified mammalian calcium channel α_1 subunits. Specifically, the following twelve mammalian α_1 subunit sequences were used to screen the *C. elegans* genomic data bank:

rat brain α_{1A}	: GTCAAAACTC AGGCCTTCTA CTGG	SEQ ID. No. 1
rat brain α_{1A}	: AACGTGTTCT TGGCTATCGC GGTG	SEQ ID. No. 2
rat brain α_{1B}	: GTGAAAGCAC AGAGCTTCTA CTGG	SEQ ID. No. 3
rat brain α_{1B}	: AACGTTTTCT TGGCCATTGC TGTG	SEQ ID. No. 4
rat brain α_{1C}	: GTTAAATCCA ACGTCTTCTA CTGG	SEQ ID. No. 5
rat brain α_{1C}	: AATGTGTTCT TGGCCATTGC GGTG	SEQ ID. No. 6
rat brain α_{1D}	: GTGAAGTCTG TCACGTTTTA CTGG	SEQ ID. No. 7
rat brain α_{1D}	: AAGCTCTTCT TGGCCATTGC TGTA	SEQ ID. No. 8
rat brain α_{1E}	: GTCAAGTCGC AAGTGTTCTA CTGG	SEQ ID. No. 9
rat brain α_{1E}	: AATGTATTCT TGGCTATCGC TGTG	SEQ ID. No. 10
rat brain consensus #1	: ATCTAYGCYR TSATYGGSAT G	SEQ ID. No. 11
rat brain consensus #2	: ATGGACAAYT TYGASTAYTC	SEQ ID. No. 12

This search identified four distinct *C. elegans* cosmids that contain open reading frames (coding regions) that exhibit homology to mammalian calcium channel α_1 subunits:

cosmid and reading frame T02C5.5

cosmid and reading frame C48A7.1

cosmid and reading frame C54D2.5

cosmid and reading frame C27F2.3

Examination of the four *C. elegans* cosmid sequences by phylogeny analysis shows that two of these, T02C5.5 and C48A7.1, correspond closely with previously identified mammalian α_1 subunits. T02C5.5 appears to be an ancestral member related to the mammalian α_{1A}, α_{1B} and α_{1E} subunits. C48A7.1 appears to be an ancestral member related to the mammalian L-type channels encoded by α_{1C}, α_{1D} and α_{1S}. In contrast, the *C. elegans* cosmids C54D2.5 and C27F2.3 identify novel types of calcium channel α_1 subunits distinct from the other mammalian subtypes.

Mammalian counterparts of the *C. elegans* calcium channel α_1 subunit encoded by C54D2.5 were identified by screening of the GenBank expressed sequence tag (EST) data bank. This analysis identified a total of 13 mammalian sequences that exhibit some degree of DNA sequence and amino acid identity to C54D2.5, of which 8 are human sequences. (Table 2) Some of these sequences appear unlikely to encode novel calcium channel subunits because they either exhibit a significant degree of homology to previously identified mammalian α_1 subunits (for example, clones H06096 and H14053) or exhibit homology in a region not considered to be diagnostic of calcium channel α_1 subunits specifically as opposed to other types of ion channel molecules in general (for example, clone D20469). One of the five remaining sequences was evaluated and appears to encode a sodium channel. Four sequences (H55225, H55617, H55223, and H55544), however, encode what are believed to be previously unidentified calcium channel α_1 subunits. For these subunits, the amino acid sequences closely match that of known calcium channel subunits in conserved regions but are sufficiently different to indicate that they do not encode previously identified mammalian calcium channel α_1 subunits, α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, or α_{1S}. The expected amino acid sequence closely matches but is not identical to amino acid sequences in these known calcium channel subunits.

Table 2

Query = C54D2.5 CE02562 CALCIUM CHANNEL ALPHA-1 SUBUNIT LG:6

Database: Non-redundant Database of GenBank EST Division

824,500 sequences; 302,742,428 total letter

Sequences producing High-scoring Segment Pairs:		Frame	Score	P(N)
gb|AA183990|AA183990	ms53e02.r1 Life Tech mouse embry...	+1	108	1.8e-24
gb|H55225|H55225	**CHR220164 Homo sapiens genomic c...**	**+1**	**136**	**2.5e-10**
dbj|D68412|CELK131B1F	C.elegans cDNA clone yk131b1 : 5...	+3	117	1.7e-06
gb|R75128|R75128	MDB1075 Mouse brain, Stratagene ...	+3	113	7.2e-06
gb|H55617|H55617	**CHR220556 Homo sapiens genomic c...**	**+2**	**102**	**2.8e-05**
emb|F07776|HSC2HD061	**H. sapiens partial cDNA sequence...**	**+3**	**100**	**0.00057**
gb|W76774|W76774	me84e08.r1 Soares mouse embryo N...	+2	98	0.0012
gb|H06096|H06096	yl77e01.r1 Homo sapiens cDNA clo...	+3	98	0.0015
gb|H14053|H14053	ym65d10.r1 Homo sapiens cDNA clo...	+2	91	0.0036
gb|H55223|H55223	**CHR220162 Homo sapiens genomic c...**	**+2**	**87**	**0.0039**
dbj|D35703|CELK024D9F	C.elegans cDNA clone yk24d9 : 5'...	+3	74	0.046
dbj|D20469|HUMGS01443	Human HL60 3'directed MboI cDNA,...	-2	66	0.91
gb|H55544|H55544	**CHR220483 Homo sapiens genomic c...**	**+1**	**65**	**0.98**

The four sequences (H55225, H55617, H55223, and H55544) are found on human chromosome 22, and are now believed to all be part of the same gene encoding the novel human calcium channel subunit α_{11}.

The sequences of the four selected sequences and the references from which they are taken are given as follows:

H55225 SOURCE human clone=C22_207 primer=T3 library=Chromosome 22 exon

Trofatter, et al., *Genome Res.* 5 (3): 214-224 (1995)

SEQ ID No. 13

1 GTGATCACTC TGGAAGGCTG GGTGGAGATC ATGTACTACG TGATGGATGC TCACTCCTTC

61 TACAACTTCA TCTACTTCAT CCTGCTTATC ATACCCCTCT TGCCTTGCAC CCCATATGGT

121 CTTCCCAGAG TGAGCTCATC CACCTCGTCA TGCCTGACTC GACGTTCA

H55617 SOURCE human clone=C22_757 primer=T3 library=Chromosome 22 exon

Trofatter, et al., *Genome Res.* 5 (3): 214-224 (1995)

SEQ ID No. 14

1 GATGGTCGAG TACTCCCTGG ACCTTCAGAA CATCAACCTG TCAGCCATCC GCACCGTGCG

61 CGTCCTGAGG CCCCTCAAAG CCATCAACCG CGTGCCCA

H55223 SOURCE human clone=C22_204 primer=T3 library=Chromosome 22 exon

Trofatter, et al, *Genome Res.* 5 (3): 214-224 (1995)

SEQ ID No. 15

1 CATGCTGGTG ATCCTGCTGA ACTGCGTGAC ACTTGGCATG TACCAGCCGT GCGACGACAT

61 GGACTGCCTG TCCGACCGCT GCAAGATCCT GCAG

H55544 SOURCE human clone=C22_651 primer=T3 library=Chromosome 22 exon

Trofatter, et al, *Genome Res.* 5 (3): 214-224 (1995)

SEQ ID No. 16

1 GTATCTCTGG TTACTTTAGT AGCCAACACT CTTGGCTACT CAGACCTTGG TCCCATTAAA

61 TCCCTGCGAA CCTTGAGAGC ACTAAGACCT CTAAGAGCTT TGTCTAGATT TGAAGGAATG

121 AGG

A search of the Sanger Genome Sequencing Center (Cambridge, U.K.) and the Washington University Genome Sequencing Center (St. Louis. MO) sequences in progress revealed a Bacterial Artificial Chromosome (BAC) sequence (bK206c7) that contained matches to the *C. elegans* cosmid open reading frame, C54D2.5, and to the four human

chromosome 22 ESTs, H55225, H55617, H55223,H55544. The C. elegans C54D2.5 cosmid sequence and the human EST sequences were then used to compare the translation of the bK206c7 BAC genomic sequence in all 6 reading frames. The analysis was performed using the graphical program Dotter (Eric Sohnhammer, NCBI). The analysis revealed a series of potential coding regions on one strand of the bK206c7 BAC sequence. These were subsequently translated in all 3 reading frames and the potential splice junctions identified. The translated sequence of this longer DNA fragment which is part of the human α_{11} subunit gene is given by Seq. ID Nos. 17 and 18.

Using the sequence information from the four EST's, a full length gene can be recovered using any of several techniques. Polynucleotide probes having a sequence which corresponds to or hybridizes with the EST sequences or a distinctive portion thereof (for example oligonucleotide probes having a length of 18 to 100 nucleotides) can be used to probe a human cDNA library for identification of the full length DNA encoding the α_{11} subunits. The process of identifying cDNAs of interest using defined probes is well known in the art and is, for example, described in International Patent Publication No. WO95/04144, which is incorporated herein by reference. This process generally involves screening bacterial hosts (e.g. *E. coli*) harboring the library plasmids or infected with recombinant lambda phage with labeled probes, e.g. radiolabeled with ^{32}P, and selection of colonies or phage which bind the labeled probe. Each selected colony or phage is grown up, and the plasmids are recovered. Human cDNAs are recovered from the plasmids by restriction digestion, or can be amplified, for example by PCR. The recovered cDNA can be sequenced, and the position of the calcium channel subunit-encoding region further refined, although neither process is not necessary to the further use of the cDNA to produce cell lines expressing the novel calcium channel subunits.

Longer portions of DNA-encoding the novel calcium channel subunits of the invention can also be recovered by PCR cloning techniques using primers corresponding to or based upon the EST sequences. Using this technique to identify relevant sequences within a human

brain total RNA preparation confirmed that the novel α_{11} calcium channel subunit is present in human brain. Subcloning of the 567 nt PCR product (Seq. ID No. 19, amino acids Seq. ID No. 20) and subsequent sequencing thereof showed that this product corresponds to the derived sequence form the bK206c7 BAC genomic sequence, the nucleotide sequence of which is given as SEQ ID No. 17 (amino acid sequence Seq. ID No. 18). The same experiment was performed using a rat brain RNA preparation and resulted in recovery of a substantially identical PCR product. (SEQ ID. No. 21). The protein encoded by the rat PCR product (SEQ ID No. 22) is 96% identical to the human PCR product (Seq. ID No. 20).

These sequences, which encode a partial subunit can be used as a basis for constructing full length human or rat α_{11} clones. Briefly, the subcloned α_{11} PCR product is radiolabeled by random hexamer priming according to standard methods (See, Sambrook , J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor Press) and used to screen commercial human brain cDNA libraries (Stratagene, La Jolla, CA). The screening of cDNA libraries follows standard methods and includes such protocols as infecting bacteria with recombinant lambda phage, immobilizing lambda DNA to nitrocellulose filters and screening under medium hybridization stringency conditions with radiolabeled probe. cDNA clones homologous to the probe are identified by autoradiography. Positive clones are purified by sequential rounds of screening.

Following this protocol, most purified cDNA's are likely to be partial sequence clones due the nature of the cDNA library synthesis. Full length clones are constructed from cDNA's which overlap in DNA sequence. Restriction enzyme sites which overlap between cDNAs are used to ligate the individual cDNA's to generate a full-length cDNA. For subsequent heterologous expression, the full-length cDNA is subcloned directly into an appropriate vertebrate expression vector, such as pcDNA-3 (Invitrogen, San Diego, CA) in which expression of the cDNA is under the control of a promoter such as the CMV major intermediate early promoter/enhancer. Other suitable expression vectors include, for example, pMT2, pRC/CMV, pcDNA3.1 and pCEP4.

Following these protocols, as described more fully in Example 4, full length mammalian α_{1G}, α_{1H} and α_{1I} calcium channel subunit cDNAs were isolated by using the 567 base pair human fragment (Seq. ID No. 19) to screen a rat brain cDNA library. Sequencing of the recovered sequences identified the three distinct classes of calcium channel subunits which have been demoninated herein as α_{1G}, α_{1H} and α_{1I} subunits. For each class of subunit, complete sequencing of the largest cDNA confirmed that it represented only a portion of the predicted calcium channel coding region. Complete sequences for the three new subunits were obtained by rescreening the rat brain cDNA library with probes derived from the partial length cDNAs to obtain overlapping segments. These segments were combined to form a complete gene by restriction digestion and ligation. The complete cDNA sequences of the rat α_{1G}, α_{1H} and α_{1I} subunits are given by Sequence ID Nos. 23, 25 and 27, respectively. Corresponding amino acid sequences are given by Sequence ID Nos. 24, 26 and 28. The same techniques are employed to recover human sequences by screening of a human or other mammalian library. Thus, for example, partial length human sequences for α_{1G} and α_{1H} T-type calcium channels have been recovered using the same probe (Seq. ID No. 19) and the full length rat α_{1I} cDNA (Seq. ID. No. 27) has been used to recover a partial length DNA encoding a human α_{1I} T-type calcium channel. The DNA and amino acid sequences for these partial length human calcium channels are given by Seq. ID Nos. 30-35.

Once the full length cDNA is cloned into an expression vector, the vector is then transfected into a host cell for expression. Suitable host cells include *Xenopus* oocytes or mammalian cells such as human embryonic kidney cells as described in International Patent Publication No. WO 96/39512 which is incorporated herein by reference and Ltk cells as described in US Patent No. 5,386,025 which is incorporated herein by reference. Transfection into host cells may be accomplished by microinjection, lipofection, glycerol shock, electroporation calcium phosphate or particle-mediated gene transfer. The vector may also be transfected into host cells to provide coexpression of the novel α_1 subunits with a β and/or an $\alpha_2\delta$ subunit.

To confirm that the three full length cDNAs (sequence ID Nos. 23, 25 and 27) encoded function calcium channels, the α_{1G} and α_{1I} cDNAs were transiently transfected into human embryonic kidney cells and evaluated using electrophysiological recording techniques. As described in more detail in Example 5 below, and as illustrated in Figs. 1-4), the results are consistent with a role of these subunits in native T-type channels in nerve, muscle and endocrine cells.

The resulting cell lines expressing functional calcium channels including the novel α_1 subunits of the invention can be used test compounds for pharmacological activity with respect to these calcium channels. Thus, the cell lines are useful for screening compounds for pharmaceutical utility. Such screening can be carried out using several available methods for evaluation of the interaction, if any, between the test compound and the calcium channel. One such method involves the binding of radiolabeled agents that interact with the calcium channel and subsequent analysis of equilibrium binding measurements including but not limited to, on rates, off rates, K_d values and competitive binding by other molecules. Another such method involves the screening for the effects of compounds by electrophysiological assay whereby individual cells are impaled with a microelectrode and currents through the calcium channel are recorded before and after application of the compound of interest. Another method, high-throughput spectrophotometric assay, utilizes the loading the cell lines with a fluorescent dye sensitive to intracellular calcium concentration and subsequent examination of the effects of compounds on the ability of depolarization by potassium chloride or other means to alter intracellular calcium levels. Compounds to be tested as agonists or antagonists of the novel α_{1I} calcium channel subunits are combined with cells that are stably or transiently transformed with a DNA sequence encoding the α_{1G}, α_{1H} and α_{1I} calcium channel subunits of the invention and monitored using one of these techniques.

DNA fragments with sequences given by SEQ ID Nos. 13-17 and 19, or polynucleotides with the complete coding sequences as given by Sequence ID Nos. 23, 25 and 27 or distinctive portions thereof which do not exhibit non-discriminatory levels of homology

with other types of calcium channel subunits may also be used for mapping the distribution of α_{1G}, α_{1H} and α_{1I} calcium channel subunits within a tissue sample. This method follows normal histological procedures using a nucleic acid probe, and generally involves the steps of exposing the tissue to a reagent comprising a directly or indirectly detectable label coupled to a selected DNA fragment, and detecting reagent that has bound to the tissue. Suitable labels include fluorescent labels, enzyme labels, chromophores and radio-labels.

EXAMPLE 1

In order to isolate novel human calcium channel α_1 subunits using standard molecular cloning protocols, synthetic DNA probes are prepared, radiolabeled with ^{32}P and utilized to screen human cDNA libraries commercially available in lambda phage vectors (Stratagene, La Jolla, CA) based on the human DNA sequences for H55225, H55617, H55223, and H55544. DNA fragments with the sequence of sequence ID Nos 17 and 19 may also be used for this purpose. Positive phage are purified through several rounds of screening involving immobilizing the phage DNA on nitrocellulose filters, hybridizing with the radiolabeled probe, washing off of excess probe and then selection of clones by autoradiography. Clones identified by this approach are expected to be partial length clones due to the nature of cDNA library synthesis and several rounds of screening for each calcium channel type may be necessary to obtain full-length clones.

To characterize the clones, double stranded plasmid DNA is prepared from the identified clones and the sequences are determined using ^{35}S dATP, Sequenase and standard gel electrophoresis methods. Regions of similarity and regions of overlap are determined by comparison of each cDNA sequence.

Full-length clones are constructed by ligating overlapping cDNA fragments together at common restriction enzyme sites. The full-length clones are subsequently inserted into vectors suitable for expression in vertebrate cells (e.g. pMT2, pRC/CMV, pcDNA3.1, pCEP4,

pREP7) by ligation into restriction sites in the vector polylinker region which is downstream of the promoter used to direct cDNA expression.

DNA encoding the novel calcium channels can be stably or transiently introduced into eukaryotic cells (e.g. human embryonic kidney, mouse L cells, chinese hamster ovary, etc) by any number of available standard methods. Stable transfection is achieved by growing the cells under conditions that promote growth of cells expressing a marker gene which is contained in the expression vector (e.g. dihydrofolate reductase, `thymidine kinase, or the like). The heterologous DNA encoding the human calcium channel may be integrated into the genome or may be maintained as an episomal element.

Expression of the human calcium channel in transfected cells may monitored by any number of techniques, including Northern blot for RNA analysis, Southern blot for cDNA detection, electrophysiological assay for calcium channel function, the binding of radiolabeled agents thought to interact with the calcium channel, and fluorescent assay of dyes sensitive to intracellular calcium concentration.

EXAMPLE 2

Heterologous Expression of Mammalian α_{11} Calcium Channels in Cells

A. Transient Transfection in Mammalian Cells

Host cells, such as human embryonic kidney cells, HEK 293 (ATCC# CRL 1573) are grown in standard DMEM medium supplemented with 2 mM glutamine and 10% fetal bovine serum. HEK 293 cells are transfected by a standard calcium-phosphate-DNA co-precipitation method using a full-length mammalian α_{11} calcium channel cDNA (for example, Seq. ID. No. 27) in a vertebrate expression vector (for example see Current protocols in Molecular Biology). The α_{11} calcium channel cDNA may be transfected alone or in combination with other cloned subunits for mammalian calcium channels, such as $\alpha2\delta$ and β subunits, and also with clones for marker proteins such the jellyfish green fluorescent protein.

Electrophysiological Recording: After an incubation period of from 24 to 72 hrs the culture medium is removed and replaced with external recording solution (see below). Whole cell patch clamp experiments are performed using an Axopatch 200B amplifier (Axon Instruments, Burlingame, CA) linked to an IBM compatible personal computer equipped with pCLAMP software. Microelectrodes are filled with 3 M CsCl and have typical resistances from 0.5 to 2.5 MΩ. The external recording solution is 20 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 40 mM TEACl, 10 mM Glucose, 65 mM CsCl, (pH 7.2). The internal pipette solution is 105 mM CsCl, 25 mM TEACl, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES (pH 7.2). Currents are typically elicited from a holding potential of -100 mV to various test potentials. Data are filtered at 1 kHz and recorded directly on the harddrive of a personal computer. Leak subtraction is carried out on-line using a standard P/5 protocol. Currents are analyzed using pCLAMP versions 5.5 and 6.0. Macroscopic current-voltage relations are fitted with the equation $I = \{1/(1+\exp(-(V_m-V_h)/S)\} \times G - (V_m-E_{rev})$, where V_m is the test potential, V_h is the voltage at which half of the channels are activated, and S reflects the steepness of the activation curve and is an indication of the effective gating charge movement. Inactivation curves are normalized to 1 and fitted with $I = (1/1 + \exp((V_m-V_h)/S)$ with V_m being the holding potential. Single channel recordings are performed in the cell-attached mode with the following pipette solution (in mM): 100 $BaCl_2$, 10 HEPES, pH 7.4 and bath solution: 100 KCl, 10 EGTA, 2 $MgCl_2$, 10 HEPES, pH 7.4.

B. Transient Transfection in Xenopus Oocytes

Stage V and VI Xenopus oocytes are prepared as described by Dascal et al (1986), Expression and modulation of voltage-gated calcium channels after RNA injection into Xenopus oocytes. Science 231:1147-1150. After enzymatic dissociation with collagenase, oocytes nuclei are microinjected with the human α_{11} calcium channel cDNA expression vector construct (approximately 10 ng DNA per nucleus) using a Drummond nanoject apparatus. The α_{11} calcium channel may be injected alone, or in combination with other mammalian

calcium channel subunit cDNAs, such as the $\alpha 2$-δ and $\beta 1b$ subunits. After incubation from 48 to 96 hrs macroscopic currents are recorded using a standard two microelectrode voltage-clamp (Axoclamp 2A, Axon Instruments, Burlingame, CA) in a bathing medium containing (in mM): 40 $Ba(OH)_2$, 25 TEA-OH, 25 NaOH, 2 CsOH, 5 HEPES (pH titrated to 7.3 with methan-sulfonic acid). Pipettes of typical resistance ranging from 0.5 to 1.5 mΩ are filled with 2.8M CsCl, 0.2M CsOH, 10mM HEPES, 10mM BAPTA free acid. Endogenous Ca (and Ba) -activated Cl currents are suppressed by systematically injecting 10-30 nl of a solution containing 100mM BAPTA-free acid, 10mM HEPES (pH titrated to 7.2 with CsOH) using a third pipette connected to a pneumatic injector. Leak currents and capacitive transients are subtracted using a standard P/5 procedure.

EXAMPLE 3

Construction of Stable Cell Lines Expressing Mammalian α_{11} Calcium Channels

Mammalian cell lines stably expressing human α_{11} calcium channels are constructed by transfecting the α_{11} calcium channel cDNA into mammalian cells such as HEK 293 and selecting for antibiotic resistance encoded for by an expression vector. Briefly, a full-length mammalian α_{11} calcium channel cDNA (for example Seq. ID No. 27) subcloned into a vertebrate expression vector with a selectable marker, such as the pcDNA3 (InvitroGen, San Diego, CA), is transfected into HEK 293 cells by calcium phosphate coprecipitation or lipofection or electroporation or other method according to well known procedures (Methods in Enzymology, Volume 185, Gene Expression Technology (1990) Edited by Goeddel, D.V.). The α_{11} calcium channel may be transfected alone, or in combination with other mammalian calcium channel subunit cDNAs, such as the $\alpha 2$-δ and $\beta 1b$ subunits, either in a similar expression vector or other type of vector using different selectable markers. After incubation for 2 days in nonselective conditions, the medium is supplemented with Geneticin (G418) at a concentration of between 600 to 800 ug/ml. After 3 to 4 weeks in this medium, cells which are resistant to G418 are visible and can be cloned as isolated colonies using standard cloning

rings. After growing up each isolated colony to confluency to establish cell lines, the expression of α_{1I} calcium channels can be determined at with standard gene expression methods such as Northern blotting, RNase protection and reverse-transcriptase PCR.

The functional detection of α_{1I} calcium channels in stably transfected cells can be examined electrophysiologically, such as by whole patch clamp or single channel analysis (see above). Other means of detecting functional calcium channels include the use of radiolabeled ^{45}Ca uptake, fluorescence spectroscopy using calcium sensitive dyes such as FURA-2, and the binding or displacement of radiolabeled ligands that interact with the calcium channel.

EXAMPLE 4

In order to recover full-length mammalian sequences for novel calcium channels, the 567 base pair partial length human brain α_{1I} cDNA was gel-purified, radio-labelled with ^{32}P dATP and dCTP by random priming (Feinberg et al., 1983, *Anal. Biochem.* 132: 6-13) and used to screen a rat brain cDNA library constructed in the phase vector Lambda Zapp II. (Snutch et al., 1990, *Proc Natl Acad Sci (USA)* 87: 3391-3395). Screening was carried out at 62°C in 5XSSPE (1XSSPE= 0.18 M NaCl; 1mM EDTA; 10 mM sodium phosphate, pH=7.4 0.3% SDS, 0.2 mg/ml denatured salmon sperm DNA). Filters were washed at 62°C in 0.2X SSPE/0.1% SDS. After three rounds of screening and plaque purification, positive phages were transformed into Bluescript phagemids (Stratagene, La Jolla, CA) by *in vivo* excision.

Double stranded DNA sequencing on the recombinant phagemids was performed using a modified dideoxynucleotide protocol (Biggin et al., 1983, *Proc Natl Acad Sci (USA)* 80:3963-3965) and Sequenase version 2.1 (United States Biochemical Corp.). DNA sequencing identified three distinct classes of calcium channel α_1 subunits: designated as α_{1G}, α_{1H} and α_{1I} calcium channel subunits.

For each class of calcium channel α_1 subunit, the largest cDNA was completely sequenced and determined to represent only a portion of the predicted calcium channnel

coding region. In order to isolate the remaining portions of α_{1G} and α_{1I} calcium channel subunits, the α_{1G} clone was digested with HindIII and SpeI. The resulting 540 base pair fragment was gel purified, radiolabeled with ^{32}P dATP and dCTP by random priming and used to rescreen the rat brain cDNA library as described above. The sequence of the 540 base pair α_{1G} screening probe used is given by Seq. ID No. 29. Other sequences spanning regions of distinctiveness within the sequences for the subunits could also be employed.

Double-stranded DNA sequencing of the purified recombinant phagemids showed that additional α_{1G}, α_{1H} and α_{1I} calcium channel subunit cDNAs overlapped with the original partial length cDNAs and together encoded complete protein coding regions as well as portions of their respective 5' and 3' non-coding untranslated regions.

To recover further human sequences for the novel α_{1G} and α_{1H} calcium channels, the 567 base pair partial length human brain α_{1I} cDNA (Seq. 19) was radio-labelled with ^{32}P dATP and dCTP by random priming and used to screen a commercial human thalamus cDNA library (Clontech). Hybridization was performed overnight at 65 °C in 6 X SSPE; 0.3% SDS; 5X Denhardt's. Filters were washed at 65 °C in 0.1 X SSPE/ 0.3% SDS. After four rounds of screening and plaque purification, positive phages were selected, DNA prepared and the insert cDNA excised form the lambda vector by digestion with Eco R1 restriction enzyme. The excised cDNA was subcloned into the plasmid Bluescript KS (Stratagene, La Jolla, CA) and the DNA sequence determined using a modified dideoxynucleotide protocol and Sequenase version 2.1. The partial length α_{1G} cDNA isolated consisted of 2212 base pairs of which 279 base pairs were 5' noncoding and 1,933 base pairs were coding region representing 644 amino acids (Seq. ID Nos. 30, 31). The partial α_{1H} cDNA isolated consisted of 1,608 base pairs of which 53 base pairs were 5' noncoding and 1,555 were coding region representing 518 amino acids (Seq. ID Nos. 32, 33).

To recover further human sequences for the novel α_{1I} calcium channel, the full-length rat brain $\alpha 1I$ cDNA (Seq. 27) was radio-labelled ^{32}P dATP and dCTP by random priming and used to screen a commercial human hippocampus cDNA library (Stratagene). Hybridization

was performed overnight at 65 °C in 6 X SSPE; 0.3% SDS; 5X Denhardt's. Filters were washed at 65 °C in 0.1 X SSPE/ 0.3% SDS. After four rounds of screening and plaque purification, positive phages were transformed into Bluescript phagemids (Stratagene, LA Jolla, CA) by *in vitro* excision. The excised cDNA DNA sequence was determined using a modified dideoxynucleotide protocol and Sequenase version 2.1. The partial α_{1I} cDNA isolated consisted of 1,080 base pairs of coding region representing 360 amino acids (Seq. ID Nos. 34, 35).

EXAMPLE 5

Double-stranded DNA sequencing of the purified recombinant phagemids showed that additional α_{1G} and α_{1I} calcium channel cDNAs overlapped with the original partial length cDNAs and together encoded complete protein coding regions as well as portions of their respective 5' and 3' non-coding untranslated regions. (Seq. ID Nos. 23 and 27, respectively) In addition to the α_{1G} and α_{1I} calcium channel classes, DNA sequencing of the recombinant phagemids also identified a third class of calcium channel α_1 subunit: designated as the α_{1H} calcium channel subunit. The partial length α_{1H} calcium channel cDNAs overlapped and together encoded a complete α_{1H} coding region as well as portions of the 5' and 3' untranslated regions (Seq. ID. No. 25).

Electrophysiological studies were performed on transiently-transfected human embryonic kidney cells (HEK-tsa201) prepared using the general protocol of Example 2. Transfection was carried out by standard calcium phosphate precipitation. (Okayama et al., 1991, *Methods in Molec. Biol.*, Vol. 7, ed. Murray, E.J.). For maintenance, HEK-tsa201 cells were cultured until approximately 70% confluent, the media removed and cells dispersed with trypsin and gentle trituration. Cells were then diluted 1:10 in culture medium (10% FBS, DMEM plus L-glutamine, pen-strp) warmed to 37°C and plated onto tissue culture dishes. For transient transfection, 0.5 mM $CaCl_2$ was mixed with a total of 20 μg of DNA (consisting of 3μg of either rat brain α_{1G} or α_{1I} calcium channel cDNA, 2 μg of CD8 plasmid marker, and

15 μg of Bluescript plasmid carrier DNA). The DNA mixture was mixed thoroughly and then slowly added dropwise to 0.5 ml of 2 times HeBS (274 mM NaCl, 20mM D-glucose, 10mM KCl, 1.4 mM Na_2HPO_4, 40 mM Hepes (pH=7.05). After incubation at room temperature for 20 min, 100 μl of the DNA mixture was slowly added to each dish of HEK-tsa201 cells and then incubated at 37°C for 24 to 48 hours in a tissue culture incubator (5% CO_2).

Positive transfectant cells were identified visually by addition of 1 μl of mouse CD8 (Lyt2) Dynabeads directly to the recording solution and gentle swirling to mix. Whole cell patch clamp readings were carried out with an Axopatch 200A amplifier (Axon Instruments) as described previously. (Zamponi et al., 1997, *Nature* 385: 442-446). The external recording solution was 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 40 mM TEA-Cl, 10 mM glucose, 92 mM CsCl, pH=7.2 with TEA-hydroxide. The internal pipette solutions was 105 mM CsCl, 25 mM TEA-Cl, 1mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES, pH 7.2 with NaOH.

For determination of current-voltage (I-V) relationships, cells were held at a resting potential of -100 mV and then stepped to various depolarizing test potentials. For steady-state inactivation, cells were held at varius potentials for 20 sec. and currents recorded during a subsequent test pulse to the peak potential of the I-V. Leak currents and capacitative transients were subtracted using a P/5 procedure.

Figs. 1-4 show the results obtained for HEK cells transfected with and expressing the cDNA of sequences ID Nos. 23 and 27, which correspond to the subunits designated as α_{1G} and α_{1I}. Figs. 1A and B and 2A and B shows a comparison of the waveforms and current-voltage relationship for the two channel subunit types. In the presence of recording solution containing 2mM Ca^{2+}, both the α_{1G} and α_{1I} channel subunits exhibit activation properties consistent with native T-type calcium currents. Figs 1 A and 2A show the subunit calcium current from a cell held at -120 mV and depolarized to a series of test potentials. Figs 1B and 2B show the magnitude of the calcium current. From a holding potential of -110 mV, both channel first activate at approximately -70 mV and peak currents are obtained between -40 and -50 mV. Upon depolarization to various test potentials, the current waveforms of the α_{1G}

and α_{1I} calcium channels exhibit an overlapping pattern characteristic of native T-type channels in nerve, muscle and endocrine cells.

Fig. 3 shows steady-state inactivation profiles for the α_{1G} and α_{1I} calcium channels in HEK 293 cells transiently transformed with full length cDNAs (SEQ ID Nos 23 or 27) for α_{1G} or α_{1I} subunits. Steady state inactivation properties were determined by stepping from -120 mV to prepulse holding potentials between -120 mV and -50 mV for 15 sec.. prior to a test potential of -30 mV. The data are plotted as nnormalized whole cell current versus prepulse holding potential and show that α_{1G} exhibits a V_{50} of approximately -85 mV and α_{1I} a V_{50} of approximately -93 mV. Thus, consistent with native T-type calcium channels, both of the α_{1G} and α_{1I} calcium channels exhibit pronounced steady-state inactivation at negative potentials.

Figs. 4A-C show a comparison of the voltage-dependent deactivation profiles of the α_{1G} and α_{1I} calcium channels. KEK 293 cells were transiently transfected with either an α_{1G} or α_{1I} subunit cDNA (Seq. ID No. 23 or 27). The deactivation properties of α_{1G} were determined by stepping from a holding potential of -100 mV to -40mV for 9 msec, and then to potentials between -120 mV and -45 mV. The deactivation properties of α_{1I} were determined by stepping from a holding potential of -100 mV to -40 mV for 20 msec, and then to potentials between -120 mV and -45 mV. Both channels exhibit slow deactivation kinetics compared to typical high-threshold channels, and is consistent with the α_{1G} and α_{1I} subunits being subunits for T-type calcium channels